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Regulation of Estrogen-Dependent Transcription by the LIM Cofactors CLIM and RLIM in Breast Cancer

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Abstract

Mammary oncogenesis is profoundly influenced by signaling pathways controlled by estrogen receptor α (ERα). Although it is known that ERα exerts its oncogenic effect by stimulating the proliferation of many human breast cancers through the activation of target genes, our knowledge of the underlying transcriptional mechanisms remains limited. Our published work has shown that the *in vivo* activity of LIM homeodomain transcription factors (LIM-HD) is critically regulated by cofactors of LIM-HD proteins (CLIM) and the ubiquitin ligase RING finger LIM domain-interacting protein (RLIM). Here, we identify CLIM and RLIM as novel ERα cofactors that colocalize and interact with ERα in primary human breast tumors. We show that both cofactors associate with estrogen-responsive promoters and regulate the expression of endogenous ERα target genes in breast cancer cells. Surprisingly, our results indicate opposing functions of LIM cofactors for ERα and LIM-HDs: whereas CLIM enhances transcriptional activity of LIM-HDs, it inhibits transcriptional activation mediated by ERα on most target genes *in vivo*. In turn, the ubiquitin ligase RLIM inhibits transcriptional activity of LIM-HDs but enhances transcriptional activation of endogenous ERα target genes. Results from a human breast cancer tissue microarray of 1,335 patients revealed a highly significant correlation of elevated CLIM levels to ER/progesterone receptor positivity and poor differentiation of tumors. Combined, these results indicate that LIM cofactors CLIM and RLIM regulate the biological activity of ERα during the development of human breast cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Introduction

Estrogens play an important role in several physiologic processes, including normal reproductive cycles, bone development and osteoporosis, breast and ovarian cancers, Alzheimer's disease, and cardiovascular disease. Importantly, expression of the estrogen receptor (ER), and its downstream target gene progesterone receptor (PR), is an important prognostic indicator for increased survival and responsiveness of breast tumors to endocrine therapy (1). In general, ER expression correlates with a more differentiated, less aggressive tumor phenotype (2, 3). Consistent with these data, a specific "signature" of gene expression associated with ERα expression has been identified and shown to correlate with a luminal phenotype (4). More recently, the *ERα* (*ESR1*) gene was shown to be amplified in ~20% of breast cancers and this amplification correlated with increased survival and responsiveness to antiestrogen (tamoxifen) treatment (5).

The ER elicits its biological effects in large part through its function as a ligand-activated transcription factor. The binding of estrogen to the ligand-binding domain (LBD) induces a conformational change in ERα, which allows for dimerization and the binding of coregulatory proteins (6). Despite the advances made in the understanding of ER biology and its molecular mechanisms of action, the changes involved during tumor progression, which allow tumor cells to use ER-regulated transcription for their growth and survival, remain poorly understood. Importantly, transcriptional cofactors that modulate the ability of ER to regulate differentiation may be of particular relevance for defining new clinical diagnostic markers for determining the appropriate course of treatment for patients with ER-positive tumors.

The CLIM/LDB/NLI and RLIM/RNF12 cofactors (hereafter referred to as CLIM and RLIM, respectively) are transcriptional coregulatory proteins identified by virtue of their ability to bind to the LIM domains of nuclear LIM proteins, including LIM homeodomain (LIM-HD) transcription factors (7–12). In humans, the CLIM cofactors are encoded by two genes, *CLIM1* and *CLIM2*, which produce protein products that bear 78% identity and 89% similarity to one another. mRNA encoding CLIM2 (also called LDB1 or NLI) is ubiquitously expressed, whereas expression of CLIM1 mRNA (also called LDB2) is more regionalized (8). As our polyclonal antibodies do not distinguish between CLIM1 and CLIM2 (13), the protein products recognized by our antiserum will be referred to as CLIM. The association of CLIM is required for LIM-HDs to exert at least part of their biological and transcriptional activity (14–16). Recent studies show that the stabilization of LIM-HDs by CLIM contributes to this positive function (17, 18).

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Although CLIM has been investigated largely in the context of its interaction with the LIM-HD factors, the multiple phenotypes observed in both *Drosophila* and mouse gene deletion mutants indicate that its function is much broader (11, 19). Consistent with this, the *Drosophila* homologue of CLIM, Chip, is localized in multiple locations on polytene chromosomes (11). Furthermore, CLIM interacts with several other functionally diverse transcription factors (8, 20). The RING finger ubiquitin ligase RLIM acts as a negative regulator of LIM-HD activity (9). Importantly, RLIM interacts with and targets CLIM for degradation by the ubiquitin-proteasome pathway (13), establishing a direct connection between both cofactors. Although functions for CLIM2 in the differentiation of mammary epithelial cells have been suspected (21, 22), the role of the CLIM/RLIM protein network in breast cancer remains unknown.

In the current study, we show that the LIM cofactors CLIM and RLIM are associated with ERα in primary human breast tumors and regulate its transcriptional activity *in vivo* in an opposing fashion. As elevated CLIM expression correlates with ER positivity in human breast cancers in a significant manner, these data provide strong evidence that both CLIM and RLIM are involved in the regulation of ERα, functionally connecting the ERα and LIM-HD protein networks during breast cancer.

Materials and Methods

Cell transfection and reporter assays. MCF7 cells were kindly provided by T.C. Spelsberg (Mayo Clinic, Rochester, MN). MCF7, H1299, and U-2 OS-Tet-ERα cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin. Blasticidin S (5 mg/L) and zeocin (500 mg/L) were additionally added to the medium of the U-2 OS-Tet-ERα cells to maintain selection of the transgenes. For luciferase assays, cells were grown in 24-well plates and transfected with various combinations of the indicated plasmids or small interfering RNAs (siRNA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following transfection, cells were maintained overnight in phenol red–free DMEM containing 5% charcoal-stripped FBS and then medium was changed to serum-free/phenol red–free DMEM alone or medium containing 10^{-8} mol/L ethinyl estradiol. After 24 h of treatment, luciferase extracts were harvested with Passive Lysis Buffer (Promega) and analyzed using the Dual-Luciferase Reporter System (Promega) on a Turner TD 20/20 luminometer. Human TrueClone CLIM1, CLIM2, and RLIM expression plasmids were purchased from OriGene Technologies. Mammalian and bacterial mouse RLIM and RLIMΔRING plasmids and specific RLIM siRNA were described previously (13). phRG-TK was purchased from Promega. CLIM2-specific, RLIM, and All-Stars Negative Control siRNAs were purchased from Qiagen and short hairpin RNA vectors were purchased from Open Biosystems.

Protein-protein interaction studies, Western blot, and ubiquitination analysis. Glutathione *S*-transferase (GST) or GST fusion proteins for *in vitro* interaction and ubiquitination assays were expressed and purified using glutathione-agarose beads as previously described (23). ^{35}S-labeled proteins were produced using the TNT *in vitro* transcription and translation system (Promega) according to the manufacturer's instructions. GST fusion proteins were incubated with the appropriate ^{35}S-labeled proteins and analyzed in ubiquitination assays or GST pull-down interaction analyses as previously described (13). Coimmunoprecipitation experiments of endogenous proteins were performed as previously described (23) using protein extracts from primary tumor samples or from MCF7 cells grown in phenol red–free DMEM and treated for 15 min with 10^{-8} mol/L estrogen.

For *in vivo* ubiquitination analysis, H1299 cells were cotransfected with mammalian expression plasmids for HA-tagged ERα (1 μg), His-tagged ubiquitin (2 μg), and/or RLIM (2 μg) or the respective control plasmid(s) using Lipofectamine 2000 according to the manufacturer's instructions. Cells were harvested in lysis buffer containing 6 mol/L guanidine-HCl, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, and 10 mmol/L imidazole (pH 8.0). Ubiquitinated proteins were captured using Ni-NTA-agarose beads and analyzed by Western blot analysis using an anti-HA (HA.11) monoclonal antibody (Covance).

Quantitative chromatin immunoprecipitation analysis. Chromatin immunoprecipitation (ChIP) was performed essentially as described (24). Antibodies used for ChIP were nonspecific rabbit IgG (Ab46540, Abcam), anti-ERα (HC-20, Santa Cruz Biotechnology), RLIM, or CLIM (13). ChIP and input DNA were analyzed by quantitative real-time PCR to determine the amount of immunoprecipitated DNA. Following quantitation by real-time PCR, ChIP samples were normalized to the respective input samples and then expressed as fold enrichment by dividing the individual values by the average of the nonspecific IgG ChIP samples.

Reverse transcription-PCR and quantitative real-time PCR. RNA was prepared from MCF7 cells, which were transfected and treated as indicated using Trizol (Invitrogen). Reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR was performed using a 2× real-time PCR master mix (Applied Biosystems) on an Applied Biosystems 7500 Real-time PCR machine. For reverse transcriptions, PS2-hnRNA, cathepsin D, WISP2, CLIM2, and RLIM gene expression was normalized to a control gene (*36B4*) and expressed relative to the untreated, control-transfected samples.

Immunofluorescence staining. ER-positive human invasive ductal mammary carcinoma specimens were obtained from the Tissue Bank of the University of Massachusetts Memorial Cancer Center. Immunofluorescence costaining using antibodies directed against ERα, CLIM, and RLIM was performed essentially as described previously (5).

Tissue microarray. To analyze the prognostic relevance of CLIM expression, a breast cancer tissue microarray (TMA) containing a total of >2,000 formalin-fixed, paraffin-embedded primary breast tumor specimens with available clinical follow-up and histopathologic data was used (5). This study was approved by the University of Basel Ethics Committee. Institutional Review Board approval was obtained before samples were collected. Formalin-fixed, paraffin-embedded TMA sections were deparaffinized and subsequently subjected to an autoclave pretreatment in Tris/EDTA/citrate buffer [20 mmol/L Tris-HCl (pH 7.8), 10 mmol/L sodium citrate, 13 mmol/L EDTA] for 5 min at 120°C. Endogenous peroxidase activity was blocked by H_2O_2 [1% (v/v) in methanol]. Application of the primary antibody (anti-CLIM rabbit polyclonal antibody; concentration, 1:2,000) for 2 h at 30°C was followed by the incubation with peroxidase-labeled EnVision polymer-coupled goat anti-rabbit immunoglobulins (DakoCytomation) for 20 min at 30°C. 3,3′-Diaminobenzidine was used as chromogen. Sections were counterstained with Mayer's hemalaun solution (Merck) and permanently mounted. For negative control, the primary antibody was omitted.

Staining results were judged by a board-certified pathologist (L.R.). For each tissue sample, the fraction of immunostained tumor cells was recorded, and the staining intensity was estimated on a four-step scale (0, 1, 2, 3). Tumors were then initially categorized according to arbitrarily predefined criteria into four groups, including completely negative, strongly positive, and two intermediate groups similarly as described previously (5). The exact criteria for these groups were as follows: negative (no staining at all), weak (1+ staining in ≤50% of cells or 2+ staining in ≤20% of cells), moderate (1+ staining in >50% of cells or 2+ staining in >20% but ≤70% of cells or 3+ staining in ≤30% of cells), and strong (2+ staining in >70% of cells or 3+ staining in >30% of cells). Statistical analyses of TMA data were done with the help of the Statistical Package for the Social Sciences software for PC (version 11 for Windows). *P* values of <0.05 were considered statistically significant.

Results

CLIM and RLIM colocalize with ERα in the nucleus. Because CLIM2 has been suspected to contribute to the development of breast cancer (21, 22) and CLIM2 and RLIM display a remarkably similar developmental expression profile (9, 25), we examined the

expression of both cofactors in primary human breast cancers. Immunofluorescence experiments using specific antisera directed against CLIM and RLIM revealed that both cofactors are highly expressed in all 10 human primary breast tumors examined (Fig. 1*A* and *B*; data not shown). As these tumors were characterized as ER positive, we tested whether LIM cofactors CLIM and RLIM colocalized with ERα. Indeed, we detected colocalization in the nuclei of all primary human breast tumors (Fig. 1*A* and *B*), opening the possibility that LIM cofactors CLIM and/or RLIM bind to ERα during breast cancer.

To establish an *in vitro* system for analyzing a potential role for CLIM and RLIM in the regulation of ERα activity, we tested whether these proteins also colocalize with ERα in the MCF7 breast cancer cell line. As observed in the primary tumors, CLIM and RLIM colocalized with ERα in the nucleus of MCF7 cells (Fig. 1*C*).

CLIM and RLIM interact with ERα *in vivo*. We next tested whether CLIM and RLIM are physically associated with ERα by performing coimmunoprecipitation analysis of protein extracts from primary tumor samples (Supplementary Fig. S1) and MCF7 cells (Fig. 1*D*). As previously reported for other cell lines (13), we detected an interaction between CLIM and RLIM in MCF7 cells as well as in primary tumor protein extracts. Interestingly, immunoprecipitation of CLIM or RLIM coprecipitated ERα as well. Importantly, immunoprecipitation of ERα also coprecipitated CLIM and RLIM, confirming that CLIM and RLIM indeed interact with ERα. It is, however, unclear whether ERα, RLIM, and CLIM form a ternary complex or whether the interactions of ERα with CLIM and RLIM are mutually exclusive.

Coregulators of nuclear hormone receptor (NR) transcriptional activity are known to frequently bind to a hydrophobic cleft of the LBD through receptor interaction domains (RID), which often contain LxxLL (26, 27), FxxLL (28), or LxxIL (29) motifs. Because we identified a highly conserved sequence that corresponds to a consensus RID motif within the LDB/Chip conserved domain (LCCD) of human CLIM proteins (amino acids 209–213 of hCLIM2 and 206–210 of hCLIM1; Fig. 2*A, top*), we tested whether this motif mediates a direct interaction with ERα. Indeed, using GST pull-down analysis, CLIM2 directly interacted with full-length ERα (Fig. 2*A, middle*) as well as the isolated LBD (Fig. 2*A, bottom*). Furthermore, this interaction required the putative RID because mutation of the leucine and isoleucine residues of the core RID to alanine (AxxAA) abolished the interaction (Fig. 2*A*).

As RLIM does not contain a consensus RID sequence, we used deletion mutants to determine if this cofactor also directly interacts with ERα. As shown in Fig. 2*B*, GST pull-down analysis revealed that RLIM directly interacted with ERα through a portion of its COOH-terminal domain. This was confirmed using a deletion mutant (RLIMΔRING) that lacks the COOH-terminal RING finger domain (Fig. 2*B, bottom*). Furthermore, like CLIM, RLIM also bound to the LBD of ERα (Fig. 2*B, middle*). Many coactivators, such as the p160 family of steroid receptor coactivator proteins, interact with many different NRs through RID domains. Therefore, we also tested whether the interactions of CLIM and RLIM with ERα are specific or if they are more general NR-interacting proteins. Surprisingly, CLIM and RLIM specifically interacted with ERα but not with ERβ (Fig. 2*C*) and the androgen (AR) or glucocorticoid (GR) receptors (Fig. 2*D*). Combined, these data show that the LIM cofactors CLIM and RLIM are in a complex with ERα and suggest that they may be involved in the regulation of ERα during the development of breast cancer.

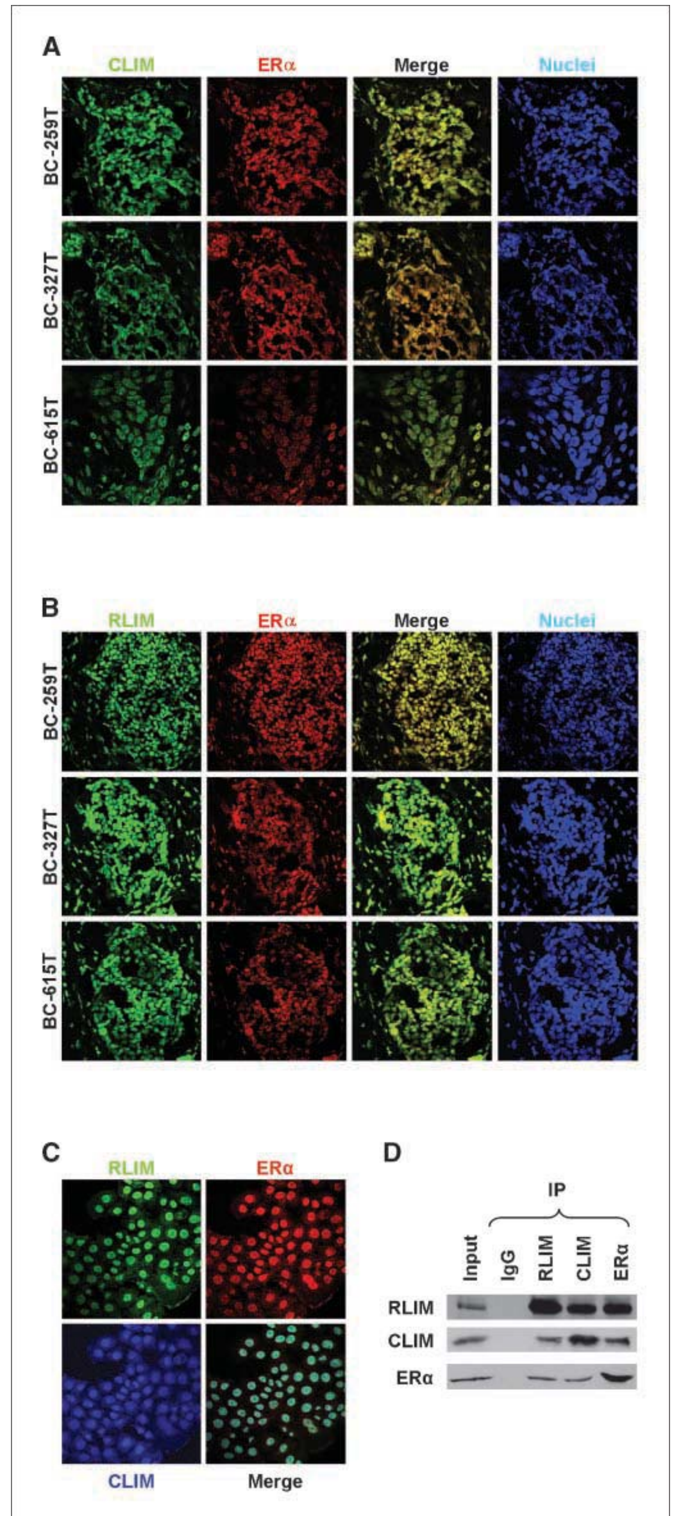

Figure 1. CLIM and RLIM are colocalized with ERα in the nucleus of breast cancer cells. Immunohistochemical staining of breast tumor samples was performed on cryosections using an anti-ERα monoclonal antibody together with anti-CLIM (*A*) or anti-RLIM (*B*) polyclonal antibodies. CLIM and RLIM expression is observed almost exclusively in the nucleus and is colocalized with ERα expression. *C,* CLIM, RLIM, and ERα are also colocalized in the nucleus of MCF7 breast cancer cells. *D,* interaction of endogenous RLIM, CLIM, and ERα in MCF7 cells. Total cell extracts of MCF7 cells were immunoprecipitated (*IP*) with specific anti-RLIM, anti-CLIM, anti-ERα, or nonspecific IgG polyclonal antibodies. Western blot analysis was performed with each antibody. Twenty percent input is shown.

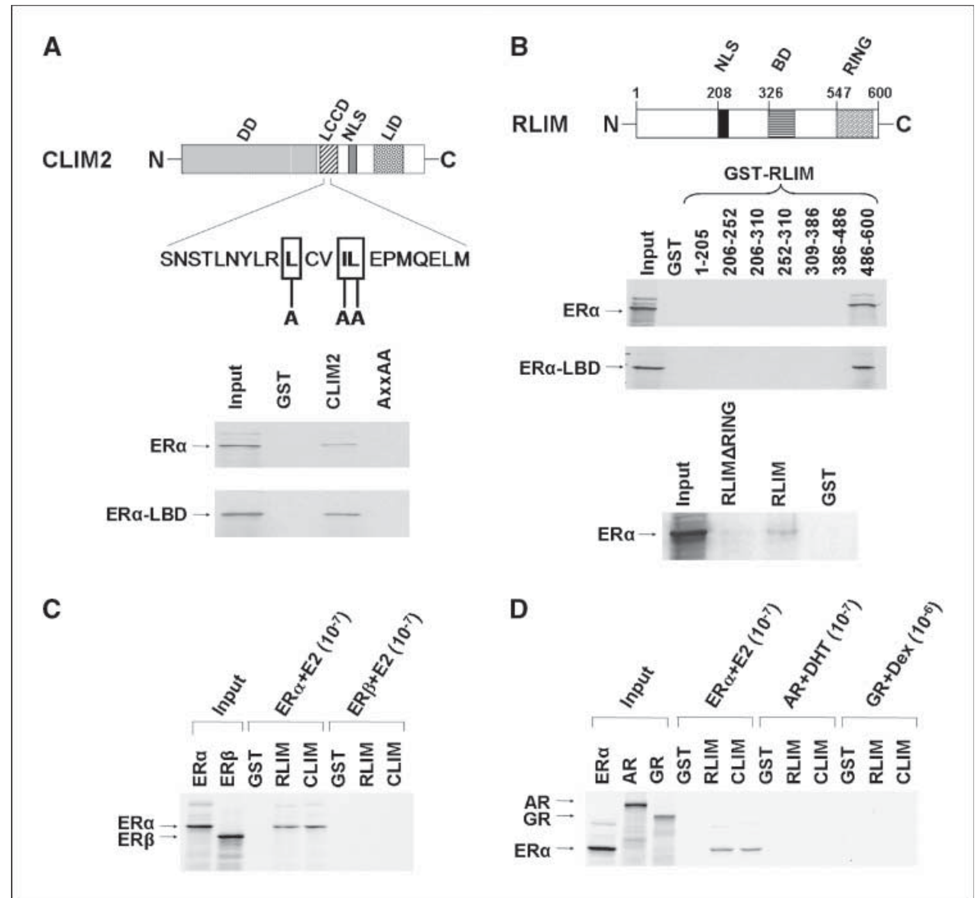

Figure 2. CLIM and RLIM are specific ERα-interacting proteins. ^{35}S-labeled *in vitro* transcribed and translated full-length human ERα (*A–D*), isolated human ERα-LBD (*A* and *B*), full-length human ERβ (*C*), full-length human AR (*D*), and full-length human GR (*D*) were tested for their ability to interact with full-length GST-CLIM2 or GST-RLIM (*A–D*) or various mutants of CLIM2 (*A*) or RLIM (*B*). Equal amounts of GST and GST fusion proteins were used and input samples of *in vitro* translated proteins were loaded separately. *A,* CLIM contains a RID (*top*) within the LCCD domain, which is necessary for interaction with full-length (*middle*) ERα and with the isolated ERα-LBD (*bottom*). *DD,* dimerization domain; *NLS,* nuclear localization signal; *LID,* LIM-interacting domain. *B,* RLIM interacts with the LBD of ERα through the COOH-terminal RING finger–containing domain. *BD,* basic domain; *RING,* RING-H2 zinc finger. CLIM2 and RLIM specifically interact with ERα but not with ERβ (*C*), AR (*D*), or GR (*D*) in the presence of the respective ligands.

ERα is a substrate for ubiquitination by RLIM. We have previously shown that RLIM negatively regulates transcription by the LIM-HD factors, at least in part, by targeting CLIM for ubiquitination and subsequent degradation by the ubiquitin-proteasome system (13). Therefore, we tested whether ERα is also a target of ubiquitination by RLIM. Indeed, using an *in vitro* ubiquitination assay, RLIM was able to induce the formation of higher molecular forms of ERα (Fig. 3*A*). We next tested whether RLIM alters the amount of high molecular weight forms of ERα *in vivo* by coexpressing ERα along with ubiquitin and RLIM. Consistent with other reports in which polyubiquitination decreases the detergent solubility of proteins, RLIM overexpression resulted in an increase in a detergent-insoluble higher molecular weight form of ERα (Fig. 3*B, bottom*). However, no decrease in the total amount of soluble or insoluble forms of ERα was observed (Fig. 3*B, middle* and *bottom*), suggesting that RLIM overexpression does not alter steady-state levels of ERα protein by targeting it for degradation by the ubiquitin-proteasome pathway. In addition, using various established assays (23), we did not find any evidence for an RLIM-mediated proteasomal degradation of ERα (data not shown). To verify that the higher molecular weight forms of ERα observed on RLIM overexpression are due to an increase in ubiquitination, we performed a His pull-down assay using extracts from cells transfected with ERα together with constructs for His-tagged ubiquitin and RLIM. Consistent with the *in vitro* results, RLIM overexpression caused a significant shift in ERα toward a higher molecular weight (Fig. 3*C*). Thus, ERα is both an *in vitro* and *in vivo* target for ubiquitination by RLIM, showing that their interaction is functional in cells.

CLIM and RLIM regulate estrogen response element–driven transcriptional activation. Based on the colocalization and interactions that we observed *in vivo* and *in vitro*, we hypothesized that CLIM and RLIM may function as transcriptional coregulators of ERα. We initially tested a potential role for these proteins in the estrogen-dependent regulation of gene expression by cotransfecting an estrogen response element (ERE)-luciferase reporter construct with CLIM2, CLIM1, or RLIM expression plasmids in MCF7 cells. As shown in Fig. 4*A*, overexpression of CLIM2 dramatically decreased reporter gene activity by more than 60% in a dose-dependent manner. CLIM1 also exerted a negative effect on ERα activity and decreased ERE-dependent transcription by >40% (Supplementary Fig. S2*A*). Mutation of the RID (AxxAA mutant) within CLIM2 completely blocked its ability to inhibit ERE-dependent transcription (Fig. 4*A*). In contrast, overexpression of RLIM increased ERα-dependent gene induction in a dose-dependent manner (Fig. 4*B*). We found that the ability of RLIM to induce transcriptional activity of ERα was dependent on its COOH-terminal domain because the RING finger–deleted RLIM mutant RLIMΔRING was no longer able to coactivate transcription (Fig. 4*B*).

To determine if endogenous CLIM and RLIM also influence ERE-dependent transcription, we cotransfected specific siRNAs against CLIM or RLIM along with an ERE-luciferase reporter construct. Using reverse transcription-PCR (RT-PCR) analysis, we were only able to detect CLIM2 but not CLIM1 mRNA expression in MCF7 cells (data not shown), consistent with previous results that show

ubiquitous CLIM2 expression but restriction of CLIM1 expression mainly to neuronal tissues (8). Based on this, we limited our CLIM siRNA studies to CLIM2. Consistent with our overexpression studies, we observed a reciprocal effect of CLIM2 and RLIM siRNAs on ERE-driven transcription. Whereas the CLIM2 siRNA increased ERE-dependent transcription ~3-fold, RLIM siRNA decreased ERE-dependent transcription ~50% (Fig. 4*C*). These results were confirmed independently using two different shRNA constructs for each protein (Supplementary Fig. S2*C*). To determine if the effects of CLIM and RLIM are specific for ERα, we tested whether their overexpression or knockdown also affected the transactivation capabilities of the GR. Consistent with our *in vitro* interaction data, no effect of CLIM and RLIM was observed on a glucocorticoid response reporter (Supplementary Fig. S2*D* and *E*). Combined, our results show that CLIM and RLIM act as specific negative and positive cofactors for ERE-dependent transcription, respectively. These data further show that the activities of both cofactors combined are able to modulate transcription from the ERE over a wide range (up to 10-fold).

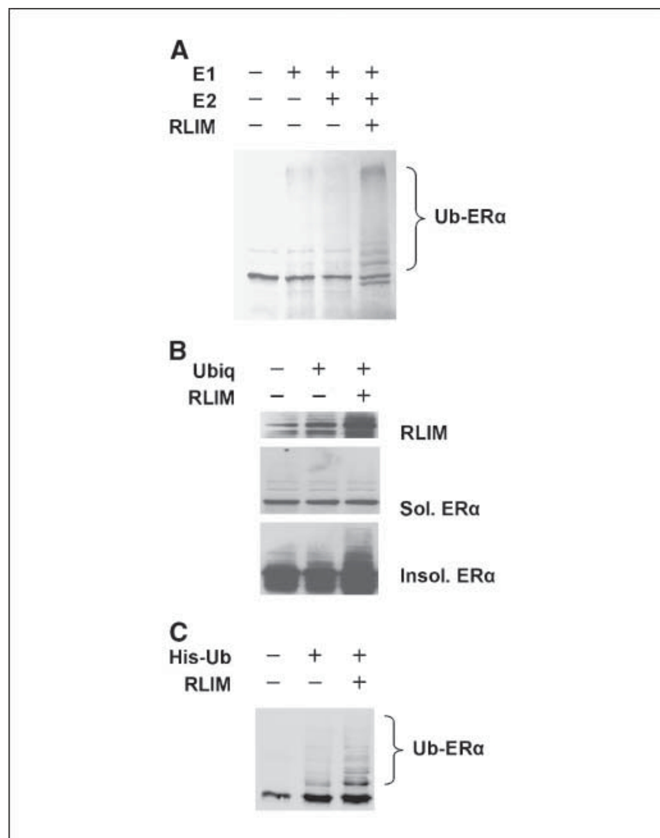

Figure 3. RLIM targets ERα for ubiquitination. *A,* ERα is an *in vitro* substrate for ubiquitination by RLIM. *In vitro* ^{35}S-labeled full-length ERα protein was incubated alone or together with various combinations of the ubiquitin activating enzyme (*E1*), the ubiquitin conjugating enzyme (*E2*) UbcH5, or bacterially expressed and purified full-length GST-RLIM. The bracket indicates higher molecular weight ubiquitinated ERα (*Ub-ERα*). *B,* overexpression of RLIM increases the formation of high molecular weight, detergent-insoluble forms of ERα. H1299 cells were transfected with ERα and ubiquitin (*Ubiq*) expression vectors as indicated. Whole extracts were prepared and detergent-soluble and detergent-insoluble fractions were separately analyzed by Western blot analysis with specific anti-RLIM polyclonal or anti-ERα monoclonal antibodies. Note that there is a noticeable increase in a high molecular weight form of ERα on overexpression of RLIM in the detergent-insoluble fraction, which is not apparent in the detergent-soluble fraction. *C,* overexpression of RLIM results in increased ERα ubiquitination in cells. H1299 cells were transfected with a His-tagged ubiquitin (*His-Ub*) expression vector with or without RLIM overexpression as in *B* and the ubiquitinated proteins were isolated using a Ni-NTA affinity matrix and analyzed by Western blot using a specific anti-ERα monoclonal antibody. The bracket indicates the increase in high molecular weight ubiquitinated ERα on RLIM overexpression.

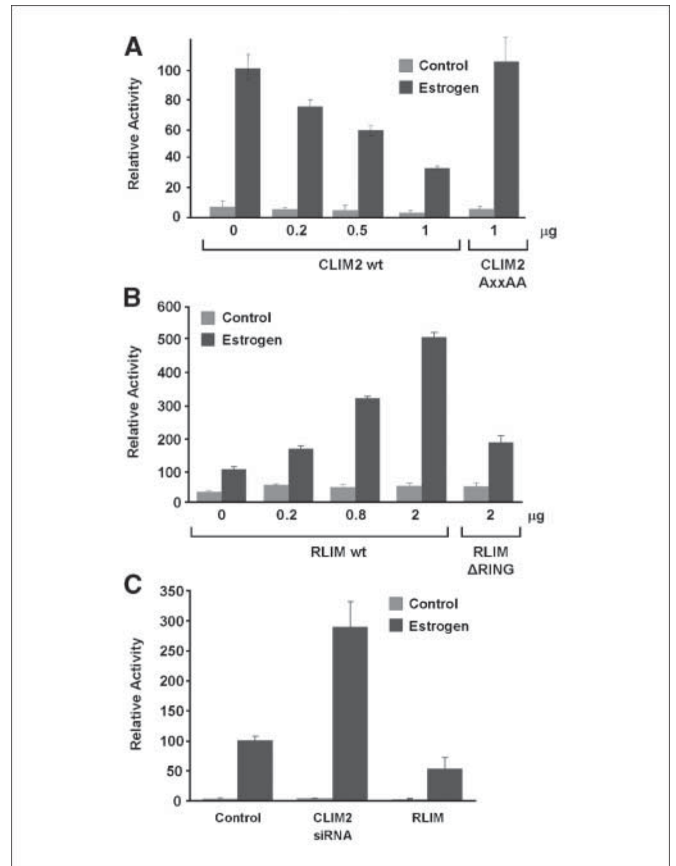

Figure 4. CLIM and RLIM regulate the transcriptional activity of ERα. *A,* CLIM overexpression dose dependently decreases ERE transcriptional activity. MCF7 cells were transfected with an ERE-containing luciferase reporter construct (ERE-TK-Luc) and an internal control plasmid for transfection efficiency (phRG-TK), which constitutively expresses *Renilla* luciferase together with the indicated amounts of a plasmid expressing CLIM2 or a CLIM2 RID mutant (AxxAA). The total amount of DNA was kept constant by adding an appropriate amount of control plasmid (pCS2). Estrogen (10^{-8} mol/L ethinyl estradiol) was added 24 h after transfection and cells were harvested after another 24 h. All transfections were normalized and expressed relative to the average of the estrogen-treated, control-transfected cells as relative activity. *B,* RLIM overexpression dose dependently increases ERE transcriptional activity. MCF7 cells were transfected with ERE-TK-Luc and phRG-TK together with the indicated amounts of a RLIM or RLIMΔRING expression vector and treated with estrogen as in *A*. The total amount of plasmid DNA was kept constant by adding the control plasmid (pCS2). *C,* CLIM2 and RLIM siRNA increase and decrease ERE activity, respectively. ERE-TK-Luc and phRG-TK were transfected as in *A* and *B* together with control, CLIM2, or RLIM siRNA. Cells were grown for 48 h to allow for a knockdown of endogenous CLIM2 or RLIM before estrogen treatment for another 24 h. Luciferase activity was expressed as relative activity as in *A* and *B*.

CLIM and RLIM regulate transcription of endogenous estrogen-regulated genes. Extensive microarray analyses have identified many estrogen-regulated genes, including *PS2* (*TFF1*), *cathepsin D*, and *WISP2*, which are directly bound by ERα at specific sites close to or within the gene and whose expression is rapidly induced following estrogen treatment (30). To determine whether CLIM2 and RLIM influence the rapid induction (2 hours after estrogen treatment) of endogenous estrogen-regulated genes, we performed quantitative real-time RT-PCR analysis of PS2

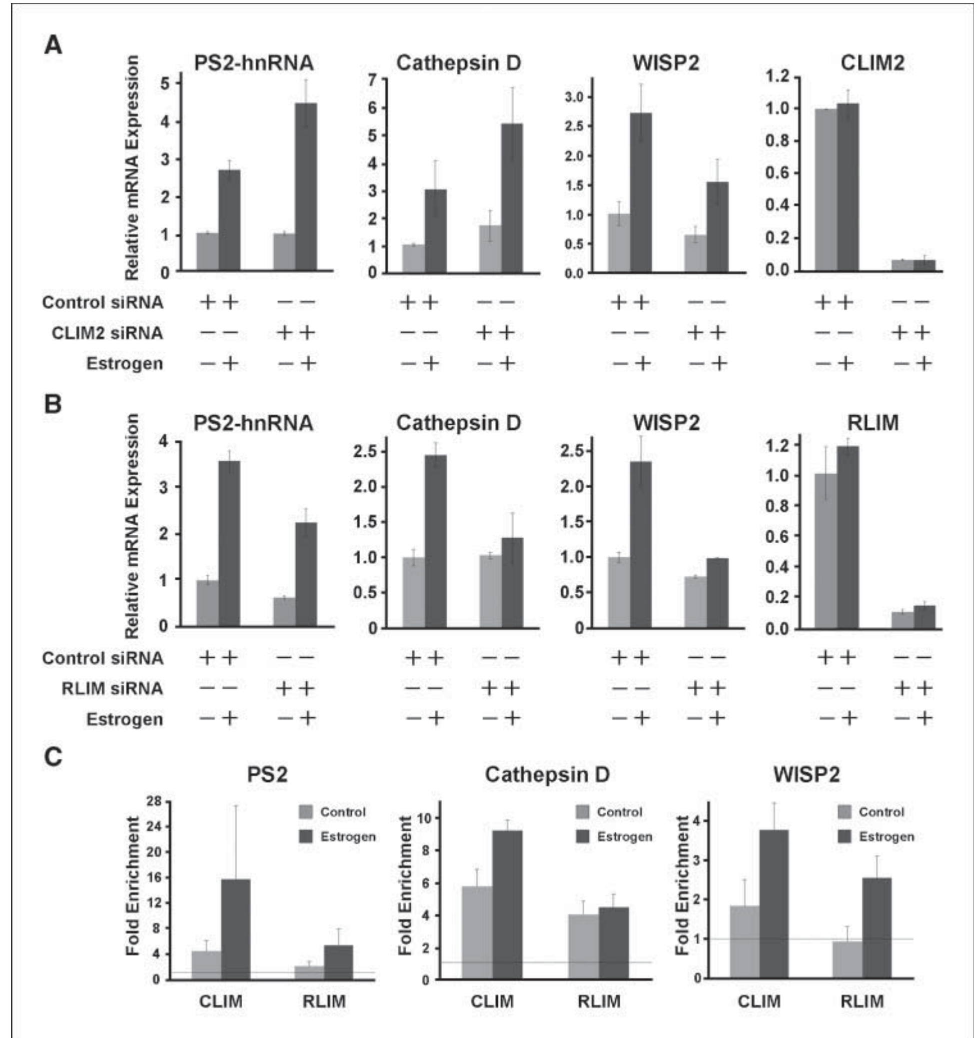

Figure 5. CLIM2 and RLIM regulate endogenous ER activity. *A,* endogenous CLIM2 regulates ERα activity in cells. MCF7 breast cancer cells were transfected with control siRNA (*A* and *B*), CLIM2 siRNA (*A*), or RLIM siRNA (*B*). Cells were grown for 48 h and treated for 2 h with estrogen (10^{-8} mol/L ethinyl estradiol) or untreated. Gene expression was measured by quantitative RT-PCR using primers specific for PS2-hnRNA, cathepsin D, WISP2, or CLIM2 mRNA. mRNA levels were normalized to an unregulated gene (*36B4*) and expressed relative to the uninduced control siRNA-transfected cells. CLIM2 mRNA levels are shown to verify efficient knockdown. *B,* RLIM is necessary for optimal induction of gene expression by the endogenous ER. MCF7 cells were transfected with RLIM siRNA and treated as in *A*. In opposition to CLIM2 siRNA, RLIM siRNA significantly decreases the induction of estrogen-regulated gene expression compared with the control conditions. RLIM mRNA levels are decreased ~90% on siRNA transfection. *C,* CLIM and RLIM are recruited to endogenous EREs. CLIM or RLIM recruitment to the *PS2, cathepsin D*, and *WISP2* genes was analyzed by ChIP analysis using chromatin from MCF7 cells untreated or treated with estrogen (10^{-8} mol/L ethinyl estradiol) for 1 h. Estrogen treatment increased the recruitment of both CLIM and RLIM to the *PS2* and *WISP2* genes, whereas only CLIM recruitment was also increased on the *cathepsin D* gene where RLIM was also present in an estrogen-independent manner. Nonspecific IgG was used to distinguish between specific and background binding. ChIP samples were normalized to input samples and expressed as fold enrichment relative to the average of all IgG ChIP samples.

heterogeneous nRNA (hnRNA), cathepsin D, and WISP2 mRNAs from control or estrogen-treated cells following siRNA-mediated knockdown of CLIM2 or RLIM gene expression (Fig. 5*A* and *B*). PS2 hnRNA was used because it very precisely reflects the rate of active transcription through the analysis of newly synthesized, short-lived, unspliced mRNA (31). Consistent with our luciferase data, knockdown of CLIM2 expression increased the rapid induction of both PS2 hnRNA and cathepsin D mRNA (Fig. 5*A*). Surprisingly, WISP2 induction was decreased following CLIM2 knockdown, possibly reflecting a target gene and context-specific role for CLIM2 in the regulation of ERα activity. In contrast, knockdown of RLIM expression dramatically decreased estrogen-dependent induction of PS2 hnRNA, cathepsin D, and WISP2 gene transcription (Fig. 5*B*). Notably, cathepsin D and WISP2 induction after 2 hours of estrogen treatment was almost completely lost following RLIM knockdown. These results show that the LIM cofactors CLIM and RLIM regulate the expression of the endogenous ER target genes *PS2, cathepsin D*, and *WISP2*.

Based on our observations that CLIM and RLIM directly interact with ERα and also influence gene regulation by ERα, we examined if LIM cofactors are present on endogenous estrogen-regulated genes. In addition to being highly dependent on ER for its expression, the *PS2* gene has the additional advantage that the ERE within its promoter has been identified and the composition of ERα-containing transcriptional activation complexes has been thoroughly characterized (32). Furthermore, the precise ERα binding sites in the *cathepsin D* and *WISP2* genes are also known from genome-wide ChIP-on-chip analysis (30). We therefore performed ChIP analysis of ERα, CLIM, and RLIM on the identified ERα binding sites of the *PS2, cathepsin D*, and *WISP2* genes. Importantly, both CLIM and RLIM were present on the ERα binding sites of all three genes (Fig. 5*C*) at levels significantly above background. The recruitment of CLIM to each of these sites increased concurrent with ERα (Fig. 5*C*; Supplementary Fig. S3) following estrogen treatment but not to a downstream sequence (+6 kb of the *PS2* gene; data not shown). RLIM recruitment to the *PS2* and *WISP2* genes was undetectable in the absence of estrogen treatment but significantly increased following induction. Surprisingly, RLIM was present at significant levels on the *cathepsin D* gene both before and after estrogen treatment.

CLIM expression correlates with ER/PR positivity and a less differentiated phenotype. To determine whether the expression of CLIM correlates with clinicopathologic variables in breast cancer, we performed immunohistochemical analysis using a high-density TMA analysis yielding 1,335 interpretable tumor

sample stainings. Samples were scored as negative, weak (Supplementary Fig. S4*A*), moderate (Supplementary Fig. S4*B*), or strong (Supplementary Fig. S4*C*) for CLIM expression. Most tumor samples (97.5%) displayed positive CLIM staining with slightly more than half (50.8%) displaying high CLIM staining, more than a third (38.8%) showing moderate staining, and only 7.8% and 2.5% displaying weak or no staining, respectively (Table 1). Although no significant correlation between CLIM expression and lymph node status was found, CLIM expression significantly correlated to the grade of differentiation, tumor type, tumor size, mitotic index, or patient age (Table 1; Supplementary Table S1). Elevated CLIM expression was observed in ductal compared with lobular and other tumors, in higher tumor stages, less differentiated tumors, and tumors of patients at lower age (Table 1; Supplementary Table S1). A significant correlation (P = 0.0001) between strong CLIM staining and positive staining for both ER and PR was observed (Table 1). Furthermore, the correlation was even stronger when the coexpression of ER and PR was correlated with positive CLIM staining ($P < 0.0001$; Table 1). Unfortunately, although the RLIM antibody provided specific immunohistochemical staining of cryosections and in cultured cells (see Fig. 1*A–C*), it was not functional in paraffin-embedded sections. Thus, we were unable to perform a parallel analysis of RLIM expression in this study. Combined, our data indicate that LIM cofactors CLIM and RLIM serve as transcriptional cofactors regulating the activity of ERα in human breast tumors.

Table 1. CLIM expression in relation to ER/PR scores and histological grade of primary breast tumors

Variable	Expression of CLIM				P
	Negative	+	++	+++	
Total (n = 1,335)	33	106	518	678	
Histologic grade					
G_1 (n = 354)	11 (3.1%)	33 (9.3%)	148 (41.8%)	162 (45.8%)	P = 0.005 (Kruskal-Wallis test)
G_2 (n = 474)	11 (2.3%)	38 (8%)	172 (36.3%)	253 (53.4%)	
G_3 (n = 347)	6 (1.7%)	28 (8.1%)	110 (31.7%)	203 (58.5%)	
Total (n = 1,175)	28 (2.4%)	99 (8.4%)	430 (36.6%)	618 (52.6%)	
ER					
Negative (n = 280)	16 (5.7%)	36 (12.9%)	107 (38.2%)	121 (43.2%)	P = 0.0001 (Mann-Whitney U test)
Positive (n = 987)	14 (1.4%)	61 (6.2%)	384 (38.9%)	528 (53.3%)	
Total (n = 1,267)	30 (2.4%)	97 (7.7%)	491 (38.8%)	649 (51.2%)	
PR					
Negative (n = 715)	17 (2.4%)	72 (10.1%)	288 (40.3%)	338 (47.3%)	P = 0.0001 (Mann-Whitney U test)
Positive (n = 447)	4 (0.9%)	15 (5.4%)	176 (39.4%)	252 (56.4%)	
Total (n = 1,162)	21 (1.8%)	87 (7.5%)	464 (39.9%)	590 (50.6%)	
ER/PR score					
1 (n = 280), ER^-	16 (5.7%)	36 (12.9%)	107 (38.2%)	121 (43.2%)	$P < 0.0001$ (Kruskal-Wallis test)
2 (n = 465), ER^+/PR^-	8 (1.7%)	40 (8.6%)	191 (41.1%)	226 (48.6%)	
3 (n = 426), ER^+/PR^+	2 (0.47%)	12 (2.8%)	169 (39.7%)	243 (57%)	
Total (n = 1,171)	26 (2.2%)	88 (7.5%)	467 (39.9%)	590 (50.4%)	
Total (n = 1,117)	26	90	408	593	
ER^- grading	15	32	91	109	
G_1 (n = 925)	3 (13.6%)	3 (13.6%)	8 (36.4%)	8 (36.4%)	P = 0.004
G_2 (n = 189)	7 (11.5%)	10 (16.4%)	26 (42.6%)	18 (29.5%)	
G_3 (n = 211)	5 (3.0%)	19 (11.6%)	57 (34.8%)	83 (50.6%)	
ER^+ grading	11	58	317	484	
G_1	7 (2.2%)	27 (8.7%)	131 (42.0%)	147 (47.1%)	$P < 0.0001$
G_2	3 (0.8%)	23 (5.9%)	138 (35.8%)	222 (57.5%)	
G_3	1 (0.6%)	8 (4.6%)	48 (27.9%)	115 (66.9%)	
Total (n = 1,023)	17	81	386	539	
PR^- grading	14	66	243	314	
G_1	7 (4.3%)	17 (10.4%)	75 (46%)	64 (39.3%)	P = 0.01
G_2	5 (2.1%)	24 (10.0%)	90 (37.7%)	120 (50.2%)	
G_3	2 (0.9%)	25 (10.6%)	78 (33.3%)	130 (55.3%)	
PR^+ grading	3	15	143	225	
G_1	1 (0.7%)	10 (6.9%)	62 (42.8%)	72 (49.6%)	P = 0.0094
G_2	2 (1.2%)	5 (3.0%)	57 (34.1%)	103 (61.7%)	
G_3	0 (0%)	0 (0%)	24 (32.4%)	50 (67.6%)	

Discussion

We have shown that CLIM and RLIM physically and functionally interact with ERα in human breast cancers. Indeed, CLIM protein expression significantly correlates with the expression of ERα and PR in a large cohort of clinical breast cancer samples. This is significant because although ERα and PR expression normally correlates with a more differentiated (and thus less aggressive) phenotype, the coexpression of CLIM in ERα- and/or PR-positive tumors correlates with a less differentiated (and thus more aggressive) phenotype. This observation is consistent with previous reports in which CLIM2 overexpression was shown to block the *in vitro* differentiation of various cell types, including mammary epithelial cells (12, 21). One possible scenario explaining such a correlation would be if CLIM were an estrogen up-regulated gene and/or if RLIM were an estrogen down-regulated gene. However, we did not find any indication that CLIM or RLIM mRNA or protein levels were affected by ERα (Supplementary Fig. S5), strongly suggesting that CLIM and RLIM are not ERα target genes.

The identification of CLIM cofactors as negative regulators for ERα was initially surprising as they are thought to act as positive transcriptional coregulators for LIM-HD transcription factors (15, 33). In this context, a recent report suggests that CLIM2, together with the LIM-only protein LMO4, may play a complex role as a negative regulator of *BMP7* gene transcription in MCF7 cells and the authors hypothesized that this interaction may play a role in the development of breast cancer (34). Intriguingly, BMP7 has also been reported to be repressed by estrogen (35). However, although the regulation of BMP7 was dependent on the interaction between LMO4 and CLIM2, no effect of LMO4 on ERα-dependent transcription was observed in our experiments (data not shown). Furthermore, BMP7 expression was increased by both CLIM2 knockdown and overexpression, thus indicating that the stoichiometry between CLIM2 and LMO4 is critical for the biological effect of these proteins (34). Similar results have also been obtained in developmental models of CLIM activity where stoichiometry seems to play a central role in determining the biological outcome of the LMO and CLIM proteins (36, 37). In contrast, ERE activity was stimulated by CLIM2 knockdown and inhibited by its overexpression, thus indicating that the mechanism by which CLIM2 regulates ERα-dependent transcription is different than that used in the LIM-HD network and is probably not dependent on the stoichiometry between CLIM2 and other LIM domain proteins such as LMO4.

In a related study, we have also observed that the RLIM protein shuttles between the cytoplasm and nucleus in mammary epithelial cells and regulates the expression of specific epithelial differentiation genes.[12] The finding that RLIM regulates ERα-dependent transcription in breast cancer further extends the functions of RLIM during the development and pathogenesis of the mammary gland. Indeed, we found that RLIM and CLIM2 play opposing roles in regulating ERα-dependent transcription, consistent with their reciprocal roles in regulating LIM-HD transcription factors. Whereas RLIM acts as a potent coactivator for the induction of estrogen-stimulated transcription, CLIM2 is a strong corepressor of ERα. Additional studies will be necessary to determine whether CLIM and RLIM can simultaneously interact with ERα or if these interactions are mutually exclusive. Given the fact that each factor interacts directly with the ERα LBD, it is likely that these are, in fact, mutually exclusive interactions. In addition, as one of the negative activities that RLIM exerts on LIM-HDs is the targeting of CLIM for proteasomal degradation, it is likely that the same activity may also be part of its positive regulation of ERα.

Evidence from several studies has shown that the ubiquitin-proteasome system plays an important role in the activation of transcription by several different transcription factors (38). However, the function of the proteasome in transcriptional regulation is multifaceted and may include the regulation of transcription factor availability, localization and complex formation, histone modification and chromatin remodeling, elongation, and silencing of transcription through the regulation of transcription factor half-life, depending on the timing and context (39).

In addition to classic transcriptional regulatory proteins, the ER also recruits specific ubiquitin-proteasome components to the target gene promoter during each cycle of binding (40). The ER itself is a target of ubiquitination (41, 42) and the inhibition of ubiquitin-proteasome activity blocks ER cycling and the activation of target gene expression (40, 41). Nevertheless, the precise mechanisms by which the ubiquitin-proteasome system functions in estrogen-regulated transcription remain unknown.

Similar to the ubiquitin ligases MDM2 and E6-AP, which also coactivate ERα-dependent transcription (43, 44), RLIM also possesses ubiquitin ligase activity and is a potent ERα coactivator. Interestingly, although we have been able to show potent and specific ubiquitination of ERα by RLIM *in vitro* and *in vivo*, we observed no effect of RLIM on ERα protein levels. Therefore, we hypothesize (*a*) that RLIM-mediated ubiquitination of ERα plays a role other than targeting it for proteasome-mediated degradation and/or (*b*) that the targeting of other proteins is critical for RLIM function in ERα-dependent transcription. Of particular note is that CyclinT1, a component of the P-TEFb complex, has been shown to directly interact with both ubiquitin (45) and ERα (46). Therefore, it is conceivable that RLIM coactivates ERα-dependent transcription by ubiquitinating ERα in a way that stimulates CyclinT1 binding without targeting ERα for degradation. RLIM may further increase the recruitment of the P-TEFb (CyclinT1/CDK9) complex through its direct interactions with both CDK9 and ERα.[12]

The fact that the down-regulation of CLIM and RLIM significantly increased and decreased levels of endogenous ER activity, respectively, indicates that LIM cofactors represent an important part of ERα regulation, despite the existence of many other cofactors that participate in ERα regulation (32). Indeed, in recent years, it became clear that many cofactors not only interact with a specific class of transcription factors but also often play significant roles in the regulation of numerous classes of transcription factors in a context-dependent manner (47). The identification of CLIM and RLIM as ERα cofactors connects the fields of estrogen signaling with nuclear LIM proteins and strongly suggests combinatorial usage of both cofactors by both systems. In this context, it is interesting to note that the LCCD in CLIM mediates interaction both with SSDP1 (48) and RLIM (13) and we have shown that the association of SSDP1 with CLIM inhibits RLIM binding, thereby leading to a stabilization of CLIM protein (17). Intriguingly, the RID that mediates interactions with ERα is also located in the LCCD directly adjacent

[12] C. Güngör et al., unpublished results.

to the SSDP1 binding site, thus opening the possibility for further combinatorial regulation. In addition, the identification of proteins that serve as targets for RLIM-mediated ubiquitination/degradation such as HDAC2 (49) offers additional combinatorial possibilities of transcriptional cross-regulation between these systems.

In conclusion, we have identified the LIM cofactors CLIM and RLIM as new ERα coregulatory proteins, which may play a role in the onset and/or progression of breast cancer. As such, specific therapies that target the expression or activity of these proteins may provide potential new therapies for ERα-positive breast tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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